DOPAMINE DEPLETION IN THE NUCLEUS ACCUMBENS OF RATS INDUCED BY A PHENYLALANINE- AND TYROSINE-FREE AMINO ACID MIXTURE

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Thesis Advisor Signature _____

ABSTRACT

Dopamine is an important neurotransmitter modulating neuronal mechanisms of human and animal behavior. In humans, one way to study the role of dopamine in neuronal mechanisms is by temporary depletion of dopamine synthesis and release. The depletion can be accomplished via administration of an amino acid (AA) mixture lacking the precursors to dopamine: phenylalanine (Phe) and tyrosine (Tyr). By using the same methodological approach in an animal model, this study examined the neurobiological consequences of this AA depletion on dopamine concentrations in the nucleus accumbens (NAc). Specifically, we used fast scan cyclic voltammetry (FSCV) to test whether the Phe-/Tyr- AA mixture reduced real-time dopamine transient activity in the NAc of rats. We hypothesized that administration of the mixture would reduce dopamine synthesis and lead to less dopamine available for release. Thus, we predicted that the Phe-/Tyr- AA mixture would decrease both the frequency and amplitude of dopamine transients in the NAc of rats. Data indicate that systemic administration of Tyr-/Phe- AA mixture, but not a control AA mixture containing Phe and Tyr, decreased frequency and caused a shift towards lower concentrations of dopamine transients in the NAc. We conclude that Phe-/Tyr- AA mixture decreases dopamine release in the NAc due to reduced dopamine synthesis.

INTRODUCTION

Dopamine has been shown to play a key role in stimulus-reward learning. During behavioral experiments, dopamine release occurs in response to a reward-predictive conditioned stimulus, and this response emerges as the animal learns the association between the stimulus and the reward (Flagel et al. 2011; Day and Carelli 2007). The pathway involved in reward signaling involves the ventral tegmental area (VTA), a brain area rich in dopaminergic neurons.

Projections from these VTA neurons extend into several other brain areas, including the prefrontal cortex and nucleus accumbens (NAc), where dopamine is released. These target areas of dopamine neurons have been implicated in signal-reward processes during learning. In humans, fMRI imaging has shown increased activity in the NAc in response to reward-prediction error in a monetary reward task (Knutson et al, 2003). In primates, increased firing of dopamine neurons in the VTA occurs at the presentation of a cue predicting a reward (Schultz et al. 1997). In addition, measurements taken in the NAc of rats have shown that phasic dopamine release can predict the expected magnitude of reward (Beyen, et al. 2010; Gan et al. 2010).

One way to study the role of dopamine is to block its function and observe the subsequent effects on behavior processes. There are several methods to do this, including blocking dopamine receptors, preventing dopamine synthesis, and lesioning dopamine neurons. In the past, the synthesis pathway of dopamine has been targeted for dopamine depletion. Administration of an amino-acid cocktail lacking both the dopamine precursor tyrosine (and tyrosine's precursor phenylalanine) has been shown to decrease dopamine levels in the brain as well as cause reduced release in response to amphetamine (McTavish et al. 1999). These previous studies were carried out using microdialysis in anesthetized rats or analysis of brain tissue content; however, in order to study the relationship between dopamine and behavioral tasks, measurements should be made in awake animals with a more time-sensitive method. Fast scan cyclic voltammetry allows rapid measurements of dopamine with excellent spatial and temporal resolution, with scans every 100 ms at a 100 μm sensor. FSCV has been used in previous studies to monitor dopamine transients and electrically-stimulated dopamine release in freely moving rats (Wightman and Robinson 2002; Robinson et al. 2008; Cacciapaglia et al. 2012), but it has not been used to assess the effects of acute dopamine depletion on spontaneous or stimulated dopamine release.

We hypothesized that administration of a tyrosine- and phenylalanine-free amino-acid mixture to awake rats would result in a decrease in both spontaneous transients as well as electrically stimulated dopamine release. In order to test this hypothesis, we first monitored spontaneous dopamine release by using FSCV in the NAc core of awake animals before and after systemic administration of an amino acid mixture either lacking tyrosine and phenylalanine (Tyr-/Phe- AA) or a control mixture containing tyrosine and phenylalanine (Tyr+/Phe+ AA).

METHODS

Animals

Adult, male Sprague-Dawley rats were purchased from Charles River Laboratories and weighed 330 g ± 11 g at the time of surgery (n=12). Rats were double housed until surgery, after which rats were single-housed for the remainder of the experiment. Rats were housed in a temperature (25^{o} C) and light controlled room (12h light/12h dark) and with access to food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the University of North Carolina, in accordance with the Public Health Service policy on Humane Care and Use of Laboratory Animals.

Surgery

Rats were anesthetized with 5% isofluorane and then maintained at 2% during surgery. The surgical procedure was previously described (Robinson et al. 2009). Briefly, rats were held in a stereotaxic frame (Kopf Instruments) on a heated pad. Once the skull was exposed, holes were drilled in the skull for placement of a stimulation electrode aimed at the VTA (AP: -5.1 mm and ML: +1.0 mm, DV -8.5 mm) and a guide cannula aimed above the NAc (AP: 1.8 mm and ML: +1.5 mm) in the right hemisphere, and a reference electrode in the cortex contralateral to the guide cannula. All coordinates were measured from bregma. The electrodes and cannula were

secured using stainless steel screws and dental acrylic. After surgery, rats were given ibuprofen (15 mg/kg, once/day for 3 days) and allowed 4-5 days to recover.

Fast scan cyclic voltammetry in awake rats

Spontaneously occurring dopamine transients were measured in awake animals, previously described (Robinson et al. 2011). Animals were handled 1 day prior to experiments. On the day of the experiment a carbon fiber electrode (7 μm in diameter and 80-120 μm length) was lowered into the guide cannula and a triangle waveform (-0.4 V to 1.3 V and back to -0.4 V vs Ag/AgCl reference electrode) was applied at 60 Hz for 20-30 minutes to condition the carbon fiber (Hermans et al. 2008). During experiments the same waveform was used at a frequency of 10 Hz to allow dopamine measurement every 100 ms. Electrical stimulation (60 Hz, 24 pulses and 124 μA) was delivered in the VTA in order to evoke dopamine in the NAc. Once evoked and/or spontaneous dopamine release (with signal-to-noise ratio > 5) was obtained, the carbon fiber was secured in place and voltammetric recording began. Basal level measurements to observe spontaneous dopamine release events (dopamine transients) were collected for 2 min. After collection of basal levels, an injection of the Tyr-/Phe- AA mixture or the Tyr+/Phe+ AA mixture was given i.p. and voltammetric data was collected for one hour on the same schedule. Next, a second injection of the same volume and solution was given (McTavish et al. 1999). Voltammetric data was collected using the same schedule for an additional two hours.

Amino Acid Solutions

The amino acid solutions were prepared as follows (adapted from McTavish et al. 1999). For the amino acid depletion mixture (Tyr-/Phe- AA), the following amino acids were added to 5 mL 1 N NaOH: 100 mg methionine, 200 mg threonine, 50 mg tryptophan, 350 mg lysine, 415.7 mg valine methyl ester HCL. The solution was stirred for 5 min and then the following amino

acids were added: 415.5 mg isoleucine methyl ester HCl and 623.2 mg leucine methyl ester HCl. The solution was brought to a final volume of 12.3 mL using distilled water. The pH was adjusted to physiological pH (~7.4). For the amino acid complete mixture (Tyr+/Phe+ AA), the depletion mixture was prepared to a final volume of 6.5 mL. In a separate vial, 250 mg of tyrosine methyl ester HCl and 250 mg phenylalanine were added to 9 mL of distilled water and stirred for 5 min. The two solutions were combined and stirred for an additional 10 min. The pH was adjusted to physiological pH (~7.4). After solvation, solutions were allowed to sit until time of injection. Solutions were made fresh daily. All amino acids were obtained from Sigma-Aldrich (St. Louis, Missouri). All rats were injected with a total volume 6.74 mL/kg to deliver 1 g/kg of amino acids, although the composition of the specific amino acids differed between the Tyr-/Phe- AA and Tyr+/Phe+ AA solutions.

Electrode calibration

Post experimental calibration of electrodes used during experiments was performed using known concentrations of dopamine (0.5 μM and 1.0 μM) in Tris buffer (2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4, 1.2 mM NaH_2PO_4, 15 mM TRIS HCl, 126 mM NaCl, pH=7.4), as in previous studies (Robinson et al. 2009).

Histological Analysis

After experiments, rats were anesthetized with urethane (≥1.5 g/kg) and cardiovascularly perfused with saline followed by 10% formalin in saline. Brains were removed and stored in 10% formalin followed by cryoprotectant (10% formalin, 25% sucrose, 0.9% NaCl).

Statistical Analysis

Data analysis was performed using TarHeel CV Analysis (Department of Chemistry, University of North Carolina at Chapel Hill) and SigmaPlot (Systat Sofware, San Jose, CA).

Dopamine transients were analyzed by comparison to a cyclic voltammogram template of dopamine (Robinson et al. 2003), and voltammograms that correlated with the template at $r > 0.866$ were accepted as dopamine transients. Current data from transients was converted to concentration based on data from electrode calibration and each transient's maximum concentration ($[DA]_{max}$) was determined.

RESULTS

Effect of a tyrosine- and phenylalanine-free amino acid mixture on the frequency of dopamine transients in the nucleus accumbens

We hypothesized that the frequency of spontaneous transients would decrease after administration of the Tyr-/Phe- AA mixture during the second hour after the second injection, based on previous microdialysis data (McTavish et al. 1999). **Figure 1** shows the effect of the Tyr-/Phe- and Tyr+/Phe+ AA mixtures on the frequency of dopamine transients in the NAc. In **Figure 1A**, representation of voltammetric signals obtained from a single rat are shown. Corresponding color plots indicate changes in current at each applied potential and show the characteristic oxidative and reductive current for dopamine. Concentration versus time traces demonstrate changes in dopamine concentration across time. Administration of the Tyr-/Phe- AA mixture decreased the number of transients indicated in the color plot and the concentration vs time traces during a 5-second interval compared with baseline. In contrast, administration of the Tyr+/Phe+ mixture to a separate rat did not decrease the number of transients during same time interval. **Figure 1B** shows average dopamine transients per minute at baseline (20 min) and 20 min intervals during the second hour after the second injection of either Tyr-/Phe- or Tyr+/Phe+ AA mixtures. In the Tyr-/Phe- group, the average number of transients per minute was 3.05 ± 0.89 at baseline and 0.57 ± 0.13 over the last 20 min after the second injection. In the Try+/Phe+

group, the number of transients was 1.85 ± 0.35 at baseline and 2.04 ± 0.78 during the last 20 min after the second injection. A two-way RM ANOVA revealed non-significant main effects of time and group ($p=0.1$) but a significant time by group interaction ($F_{3,30}=4.1$, $p<0.05$). Post-hoc analysis revealed a significant decrease in transient frequency from baseline in the Tyr-/Phe- group during all three intervals in the second hour after the second injection ($p<0.05$).

Effect of a tyrosine- and phenylalanine-free amino acid mixture on the concentration of dopamine transients in the nucleus accumbens

Figure 2 shows the $[DA]_{max}$ of transients in the NAc before and after either Tyr-/Phe- or Tyr+/Phe+ AA injection. **Figure 2A** shows average data for both the Tyr-/Phe- and Tyr+/Phe+ groups. Initially, the peak $[DA]_{max}$ was estimated for each transient and averaged over baseline (20 minutes) and over 20 min intervals during the second hour after the second injection. The average $[DA]_{max}$ was 33.9 ± 6.3 nM at baseline for the Tyr-/Phe- group and 22.5 ± 1.9 nM during the last 20 min of the second hour after the second injection. The Tyr+/Phe+ group had an average $[DA]_{max}$ of 23.0 ± 2.8 nM at baseline and 17.6 ± 2.5 nM during the last 20 min during the second hour after the second injection. 2-way RM ANOVA revealed a significant decrease in concentration of dopamine between baseline and the second hour after the second injection ($p<0.05$) but no significant group by time interaction ($p=0.2$). In order to further evaluate the effect of both mixtures on dopamine concentrations of individual transients, we plotted the distribution of transients across concentrations. **Figure 2B** shows the distribution of dopamine transients based on $[DA]_{max}$ both at baseline and during the last 20 min of the experiment. Administration of the Tyr-/Phe- AA mixture resulted in a shift in the distribution towards lower $[DA]_{max}$. This effect was not seen after administration of Tyr+/Phe+

mixture. Future analysis will statistically analyze the distribution curves with parametric multivariate regression analysis (Robinson et al. 2011).

In conclusion, administration of the Tyr-/Phe- AA mixture, but not the Tyr+/Phe+ AA mixture caused as significant decrease in DA transient frequency during the second hour after the second injection. Additionally, there was a shift in distribution towards lower $[DA]_{max}$ with the Try-/Phe- mixture but not the Tyr+/Phe+ mixture.

DISCUSSION

We found that administration of an amino acid mixture lacking tyrosine and phenylalanine led to a decrease in the frequency and concentration of spontaneous dopamine in the nucleus accumbens of awake rats. This effect is consistent with previous experiments involving anesthetized rats (McTavish et al., 1999).

Dopamine is synthesized from tyrosine by tyrosine hydroxylase locally in neurons in the presynaptic terminals, and stored in vesicles until a signal (either an action potential or electrical stimulation) evokes release (Kuhar et al., 1999). Previous studies have shown that administration of an amino acid mixture lacking tyrosine and its precursor phenylalanine leads to lower brain tyrosine levels and depletion of dopamine release (McTavish et al., 1999). In the present study, the concentration of spontaneous dopamine transients was recorded by using FSCV at a carbon fiber microelectrode. The observed decrease in the concentration of spontaneous dopamine transients was likely due to a lack of available tyrosine to synthesize new dopamine. Because dopamine is synthesized locally in neurons from tyrosine, after administering an amino acid cocktail lacking this amino acid and its precursor, phenylalanine, the neurons quickly use up all previously stored dopamine and are unable to synthesize more. As a result, over time, the concentration of dopamine detected decreases, because less dopamine is available for release.

Administration of the Tyr-/Phe- mixture led to a decrease in the frequency of dopamine transients as well as a shift in distribution towards lower concentrations. Because the mixture is designed to deplete the amount of dopamine available for release, the neurons would still fire when presented with the proper stimulus, but less dopamine will be released as a result. The number of dopamine transients decreased over time, indicating that after administration of the depletion mixture, the amount of dopamine released into the synapse decreased. Some transients may have been too small to reach the correlation threshold of 0.867 used to indicate the presence of dopamine by our criteria (Robinson et al. 2003), which would have resulted in a decrease in the number of transients. Administration of a control mixture did not lead to a decrease in transient frequency over time, and resulted in no shift in distribution across concentrations, indicating that availability of tyrosine and phenylalanine for synthesis of transients prevented loss of dopamine over the time course of the experiment. The frequency data agree with microdialysis results found by McTavish and colleagues in anesthetized rats (McTavish et al., 1999). They found that administration of a depletion mixture led to lower amphetamine-evoked dopamine concentrations in the NAc during the same time interval (during the second hour after the second injection). In future studies, the ability to measure individual transient events with FSCV will allow for the use of the depletion mixture to study the role of dopamine in real-time during behavior, such as attention to a conditioned cue or consumption of a reward.

Administration of either the Tyr+/Phe+ or Tyr-/Phe- AA mixtures caused a spike in the number of transients directly following injection (data not shown). The increase in transients directly following the administration of an amino acid cocktail may be due to the increase in transportation of the amino acids across the blood brain barrier or the interoceptive/subjective effects of the injection. Nevertheless, by the second hour after the second injection, Where the

Tyr-/Phe- AA mixture results in a net decrease in transient number after the second hour after the second injection, the Tyr+/Ph+ AA mixture results in the number of transients returning to basal levels.

Though no effect on average $[DA]_{max}$ was observed, administration of the Tyr-/Phe- AA mixture led to a shift in distribution of transients towards lower concentrations. This shift was not seen after administration of the Tyr+/Phe+ AA mixture. The changes in transient distribution have not been yet analyzed for statistical significance. However, the shift towards lower concentrations indicates a loss of transients with high $[DA]_{max}$ after administration of Tyr-/Phe- AA mixture.

This experiment demonstrated the use of a tyrosine-free amino acid mixture to deplete both concentration and frequency of naturally occurring dopamine transients in the nucleus accumbens of awake rats. Administration of a control mixture containing tyrosine and phenylalanine did not decrease the number or concentration of transients. Successful depletion via the mixture in rats allows us to investigate the role of dopamine in a variety of animal models of reward and reinforcement.

Appendix I: Figures

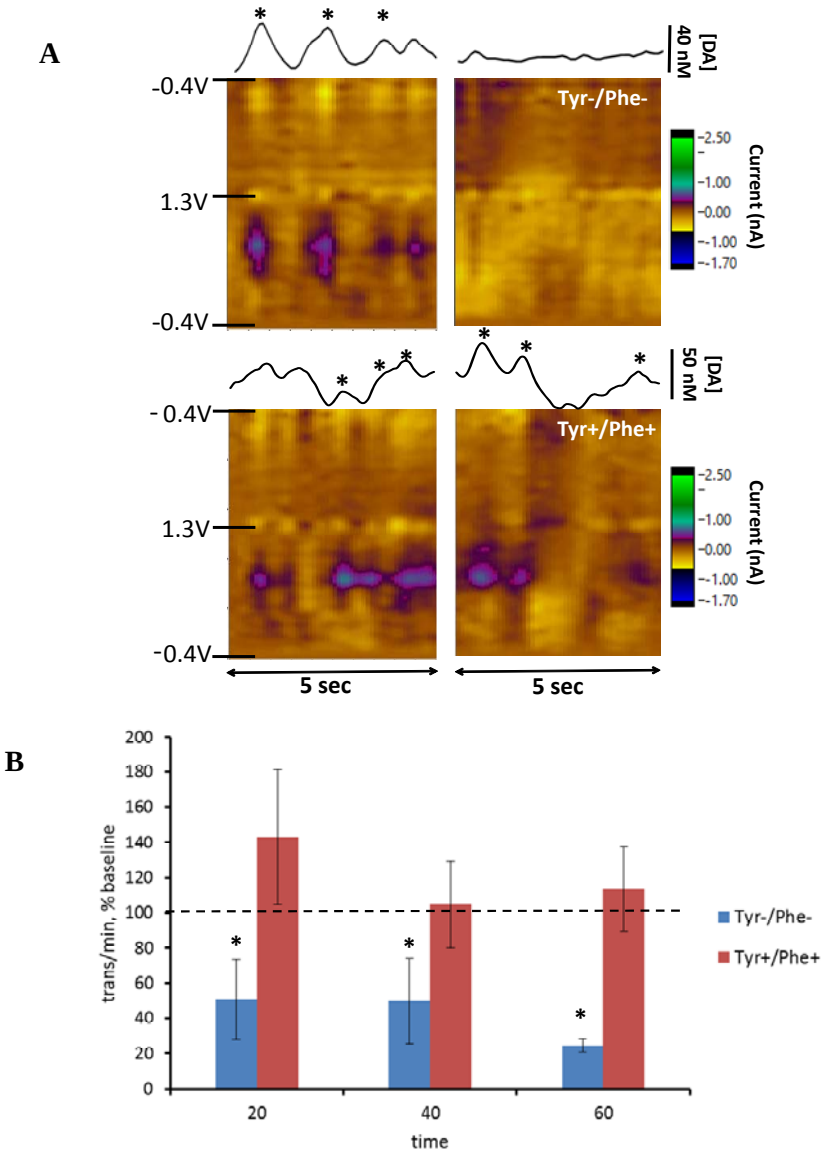

Figure 1. Effect of Tyr-/Phe- and Tyr+/Phe+ AA mixtures on frequency of spontaneous dopamine transients in NAc. **(A)** Representation of dopamine signals in the NAc of individual rat before and after Tyr-/Phe- and Tyr+/Phe+ AA mixtures. In color plots, current is expressed in color. The time is plotted on the x axis and the potential applied to the carbon fiber electrode is plotted on the *y* axis. Concentration-versus-time traces are shown above color plots with concentration converted from current after *in vitro* electrode calibration. * indicates transient found using a DA template match with $r > 0.866$. **(B)** Frequency of DA transients recorded in Tyr-/Phe- (n=6 rats) and Tyr+/Phe+ (n=6 rats) groups, presented as transients per minute in percent of baseline (indicated by the dotted line). Data are mean±SE grouped into 20-min bins for basal level (BL) and during second hour after the second injection (20, 40, 60). 2-way RM ANOVA yielded a non-significant effect of time ($p=0.1$) and a significant group X time interaction ($F_{3,30}=4.1$, $p<0.05$). * indicates significant difference from baseline. Statistical analysis was performed on raw data.

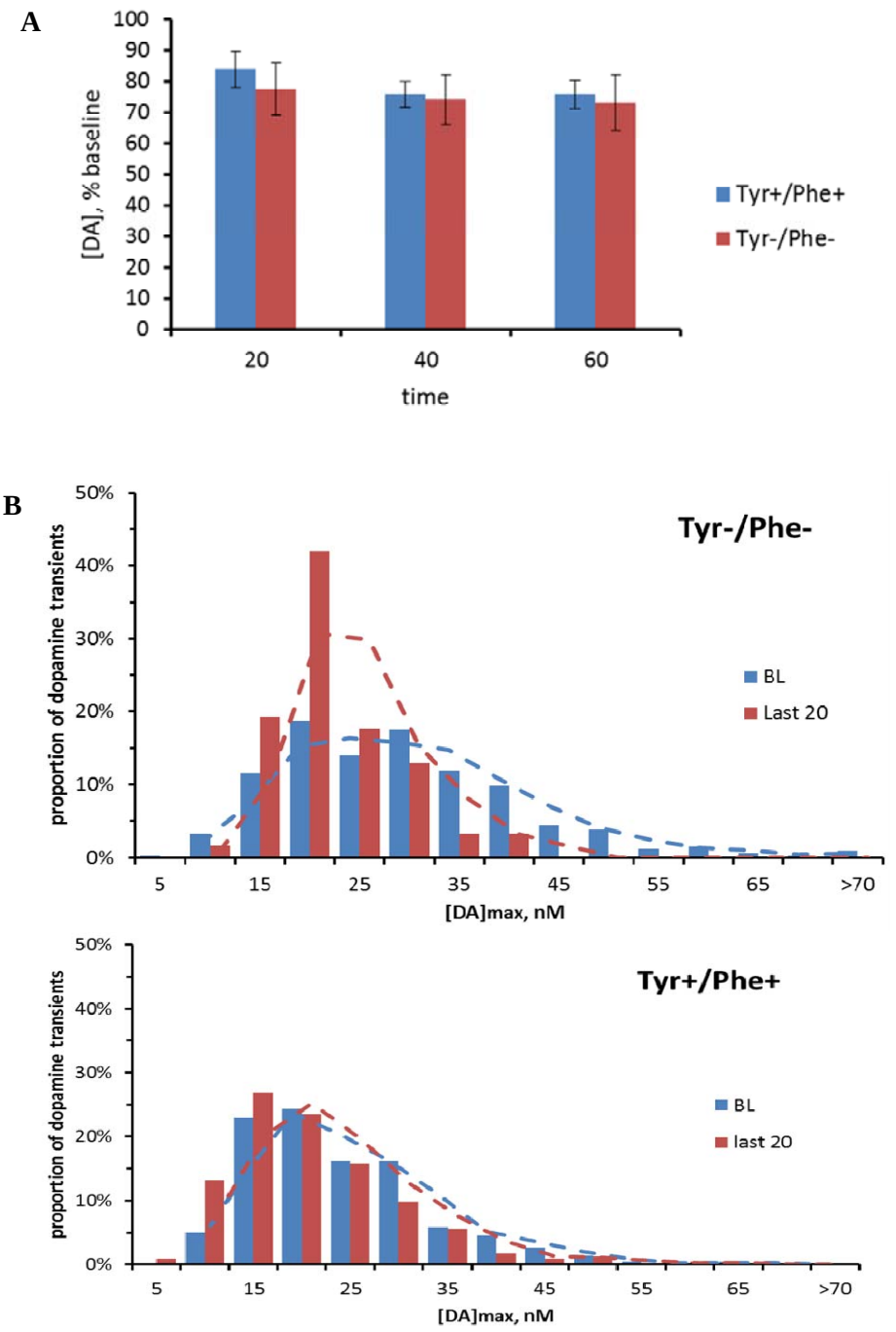

Figure 2. Effect of Tyr-/Phe- and Tyr+/Phe+ AA mixtures on concentration of spontaneous dopamine transients in NAc of rats (n=12). **(A)** Data are shown as percent of baseline in 20 min bins for both baseline (BL) and during the second hour after the second injection (20, 40, 60). 2-way RM ANOVO yielded a significant time effect (p=0.001) and a non-significant group X time interaction (p=0.2) **(B)** Distribution of transient concentrations in NAc during baseline and last 20 mins of experiment. There is a shift towards lower concentrations after administration of Tyr-/Phe- AA mixture (top) but not Try+/Phe+ AA mixture (bottom).

Appendix II: References

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